MICROALGAL DIVERSITY AND IT'S APPLICATION IN BIOREMEDIATION OF SAMBHAR SALT LAKE.

Research Protocol
For Registration in Ph.D. Programme
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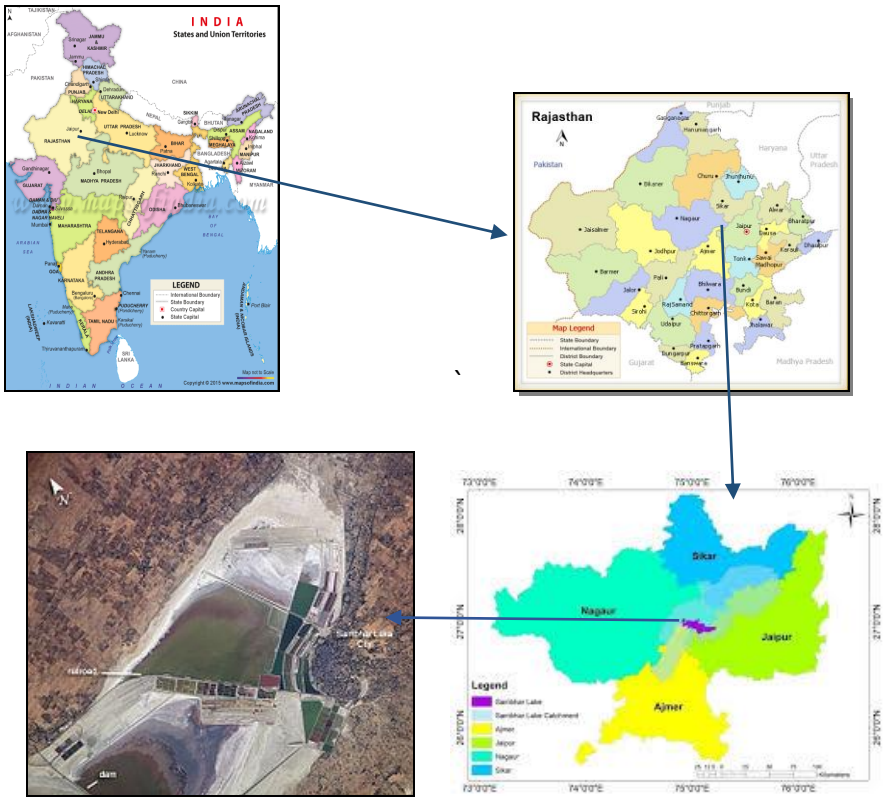
INTRODUCTION

A salt lake (saline lake) is a landlocked body of water that has a concentration of salts (typically sodium chloride) and other dissolved minerals significantly higher than other lakes. In some cases, salt lakes have a higher concentration of salt than sea water; such lakes can also be termed hyper saline lakes. The world has many famous salt lakes. Some of the well-known saline lakes that have been explored for microbial diversity, such as, The Dead sea, (Israel, Jordan), the Kenyan-Tanzanian Rift Valley lakes(Lake Magadi, Lake Bogoria, Lake Natron ,etc.); Owens Lake Texcoco, Albert Lake, etc., in the Americas; Chita and Baikal region, Loonar Lake, Tafusu Lake, Sambhar Salt Lake, etc., in Asia. Chilika Lake (Odisha) is the Asia's largest blackish water saline lake spread over 1100 sq. km. There are 6 saline lakes in India.

Sambhar lake of Rajasthan is India's largest inland salt water lake. The Sambhar Salt Lake is located 80 km southwest of the city of Jaipur (Northwest India) and 64 km northeast of Ajmer, Rajasthan. It is situated (Longitude 75°05'E, Latitude 26°58'N) in the middle of a closed depression in the Aravalli schists in Rajasthan (Figure 1). The lake basin is spread at the confluence of four districts of Rajasthan namely Jaipur, Ajmer, Nagaur and Sikar. It covers an area of 230 sq. km. Salinity of lake fluctuated from hypo to hyper saline condition (5 to 35%). Lake brine is always alkaline in nature with pH values with 7.15 to 9 (Upasani 2008).



(Figure 1. Location of Sambhar salt lake)
(Courtesy: Bhatt and Hussain, 2016).

Halophytic microorganisms are found distributed all over the world in habitats with a high concentration of salt (mainly NaCl) such as Sambhar Lake. Halophiles are salt loving microorganisms that inhabit hyper saline environments and are well equipped balance the osmotic pressure of the environment. These microorganisms resist the denaturing effects of salts.

Bioremediation is a process of decontamination of soil and aquatic systems by using plants, Algae and fungi to absorb heavy metals. It is a newer approach directed towards the treatment of decontamination. Microscopic studies of the brine samples collected from Sambhar Lake revealed the presence of some algal species. (Upasani *et al,* 2016).

The brines of Sambhar Lake are always alkaline in nature with pH values from 7.15 to 9. (Gaur *et al,* 2014). This pH is necessary for salt production and for the growth of halophytic microorganisms.

Salt production, drainage, ground water extraction and mushrooming of private salt industries, air, water, noise pollution, including human pressure and tourism are threats not only to the lake ecology but for biota to withstand the adversity (Sengupta *et al,* 2008).

The heavy metals are considered to be a serious environment problem for flora and fauna of lake and for human health. Due to anthropogenic activity the contamination of soil and aquatic system by toxic metals and other pollutants has increased in recent years (Narula *et al,* 2015).

Some of the heavy metals that are harmful for the growth of micro algae are reported such as Cadmium (Cd), Chromium (Cr), Lead (Pb), Arsenic (As), Iron (Fe), Manganese (Mn), Zinc (Zn) and Boron. These heavy metals are transformed into water along with industrial wastes and sewage.

According to BIS (Bureau of Indian Standards) these metals are present in this water at above upper permissible limit. Speedy increase in industrialization leads to deterioration in the quality of ground water and salt production in salt pans by these industries (Bhatt and Hussain, 2016).

Heavy metals cannot be degraded or destroyed because they are stable and persistent environmental contaminants. Therefore, their toxicity poses major environmental pollution and health problems and requires a constant search for detoxification of metal contaminated sites by Bioremediation.

The application of bioremediation technologies using algae to remove pollutants from the environment can be used to solve problems of pollution (Checkroun *et al,* 2013).

The microalgae which are halophytic have a remarkable ability to take up and accumulate heavy metals from their surroundings environment which can be harmful. They have ability to sequester various metal ions such as Copper, Cadmium, Nickel, and Chromium.

Halophytic Microalgae remove heavy metals directly from polluted water. This process is done by two major mechanism, the first is an active metabolism dependent uptake into their cells at low concentrations and second is bio absorption which is a non active adsorption process. The potential of halophytic microorganisms (algae, cyanobacteria, bacteria) as well as dead biomass derived from them for metal bioremediation.

The blue green algae (cyanobacteria) *Phormidium* can accumulate heavy metals like Cd, Zn, Cu, Pb and Ni. *Dunaliella salina* a halophilic green microalgae have the high tendency for Zinc accumulation and it is followed by Copper and Cobalt. The lowest tendency of accumulation was for Cadmium.

Now a days, the heavy metal pollution has become one of the most serious environmental problems throughout the world. There is a need to develop cost effective and innovative solutions to decontaminate polluted environment and to protect the ecosystem. The biological methods of metal removal such as Bioremediation were considered to minimize the effects of environmental pollution.

Huge load of waste from industries, domestic, sewage and agriculture practices find their way into river, lakes and ponds resulting in large scale deterioration of water quality leading to the unavailability of potable water. Keeping this fact the research work "Microalgal Diversity and it's application in Bioremediation of Sambhar Salt Lake." will be taken.

REVIEW OF LITERATURE

Bioremediation is a process of decontamination of soil and aquatic systems by using plants, Algae and fungi to absorb heavy metals. Bioremediation is a newer approach directed towards the treatment of decontamination. Bioremediation primarily deals with the strategies that can employ to clean up the contaminants biologically removal and recovery of heavy metals from waste water or contaminated water is important for environment protection and human health.

Microalgae strains purposefully cultivated and processed for specific bioremoval applications and have the potential to provide significant improvements in dealing with the world-wide problem in metal pollution. Microalgae have been used extensively to measure heavy metal pollution and marine environments throughout the world. In recent years, several species of the green algae have been utilized to measure heavy metal levels in many parts of the world.

Doshi *et al*, (2007) studied the use of live and dead *Spirulina* sp. for absorption of metals like Cr^{3+}, Ni^{2+}, Cu^{2+} and Cr^{6+} in form of $Cr_2O_7^{2-}$ *Spirullina* sp. treated with different metal ions have been employed to understand the sorption mechanism. It is hoped that live *Spirullina sp.* will be a strong microalgae for management of industrial waste water.

Meng, (2011) reported the biodegradation rates of linear alkyl benzene suffocate (LAS) by *Spirulina platensis* increased with Zn (II) and reached the maximum when Zn (II) was 4mg/L. The joint toxicity test showed that the combined effect of LAS and Zn (II) was synergistic. LAS can enhance the biosorption of Zn (II), and reciprocally, Zn (II) can enhance LAS biodegradation. The bioaccumulation of Cadmium in Blue Green algae *Spriullina* (*Arthrospira*) *indica* was observed by Bindiya *et al,* (2012).

Abraham and Nanda, (2010) observed the effectiveness of *Oscillatoria* was employed for the bioremediation of textile effluents. Raposo *et al,* (2010) analyzed the capacity of *Chlorella vulgaris* and the autochthonous flora of the effluents to remove some of the compounds present in the effluents. The detoxification capacity of a variety of microbes especially cyanobacteria was seen by Kannan, (2011). They collected the effluents from tannery

industry and added to the cyanobacterial growth medium in various proportion. The photosynthetic pigments and nitrogen status of *Anabena flos-aquae* were analyzed before and after the treatment with effluents. It showed that *Anabena flos-aquae* can serve as the potential bioremedial organism for industrial pollution.
Dubey *et al*, (2011) studied the biodegradation and biosorption capacity of some potential cyanobacterial species: *Oscillatoria* sp., *Synechococcus* sp., *Nodularia* sp., *Nostoc* sp. and *Cyanothece* sp. dominated the effluents and nicked cultures showed verifying sensitivity. Contaminants were removed by all the species either as individuals or in mixtures.
Bioaccumulation is the effective method for removal of heavy metal ions from waste water. Mercury is higher in *Dunaliella* algae as compared to those of Cadmium and Lead. This was studied that *Dunaliella* is highly tolerant to the ascending concentration of heavy metals and their absorption in aquatic media. This approves the usage of *Dunaliella* as useful equipment for the elimination of heavy metals environment (Imani *et al*, 2011).

Gao and Yan, (2012) observed the response of *Chara globularis* and *Hydrodictyon reticulatum* to lead pollution. Immobilization technique is essential not only in waste water treatment but also in various industries.
Use of immobilized algae in waste water treatment and heavy metal removal processes is efficient and offers significant advantages (Hammed and Ebrahim, 2007).
Murugesan *et al*, (2008) studied the biosorption process and it showed that the algae had a great potential for adsorbing the heavy metal on to the cell. The immobilized cell of *Spirulina platensis* was able to be more effective in absorbing the metal to the cell.
Shashirekha *et al*, (2008) observed the process of biosorption of trivalent chromium (Cr^{3+}) by the live culture of *Spirulina platensis* and the sorption potential by the dried biomass. In both free and immobilized states have been investigated for simulated chrome liquor in the concentration range of 100-4500 ppm. Both live and dried biomass was very good bio sorbents as they could remove high amounts of chromium from tannery waste water.
Dwivedi *et al*, (2010) reported Chromium uptake and accumulation in *Phormidium bohner.* Chekroun and Baghour (2013) reported that the blue green algae *Phormidium* successfully can hyper accumulate heavy metals like Cd, Zn, Pb, Ni, and Cu. They also reported that *Dunaliella salina* a green microalgae, have high tendency for zinc accumulation followed by Copper and Cobalt, the lowest tendency was for Cadmium, this may be due to the importance of Zinc as hydrogen transferring in photosynthesis.

Arun *et al*, (2014) observed the effect of Chromium (Cr VI) in halotolerant algae *Dunaliella salina* and *Dunaleilla tertiolecta.* The results revealed good tolerance of *Dunaliela salina* towards Chromium (VI) up to 8 ppm concentration, whereas in *Dunaliela tertiolecta* the tolerance level was up to 2 ppm concentration.
According to Narula *et al*, (2015) algae represent the best biological treatment for waste water because they increase O_2 content of water via photosynthesis and sorption of some heavy metals contaminated waters by reducing the cellular antioxidant capacity.

A number of methods has been developed for removal of heavy metals like precipitation, evaporation, ion-exchange etc. Algae are important bioremediation agents, for potential in

waste water remediation is however much wider in scope than its current role (Prabha *et al*, 2016).
The remediation of heavy metals with the metabolic potential of algae is an economic and ecofriendly measure. As other physical and chemical methods for heavy metals removal becomes ineffective at low heavy metal concentration which is not within the permissible limit of discharge in the aquatic environment, Bioremediation is the best alternative.

Verma and Sharma, (2017) observed that the industrial effluents are releasing heavy metals into the aquatic systems. Although some of the heavy metals (Co, Cu, Fe, Mn, Mo, V, Sr, Zn) are essential at trace level for the normal growth of human body as well as plants, a small increase in their threshold limit causes deadly diseases in plants, animals and in human beings, some non – essential highly toxic heavy metals (Cd, As, Pb, Hg) are characterized as potent carcinogens according to International Agency for Research on Cancers (IARC) and United States environmental protection agencies.
Vidyalaxmi *et al*, (2019) reported potential of *Dunaliella salina* for biosorption of hexavalent Chromium (VI) in aqueous solutions.
Microalgae can grow 5-10 times faster making the production rate higher compared to terrestrial biomass. *Dunaliella salina* species is considered to be one of the microalgae strains that produce a high quantity of methane using waste water substrate (Abobaker *et al*, 2019).

STATEMENT OF THE PROBLEM

Microalgal Diversity and it's application in Bioremediation of Sambhar Salt Lake.

Definition:-

1. Microalgae are microscopic, unicellular and multicellular microorganism, typically found in fresh water and marine system. These are capable of performing photosynthesis and important for life on earth.
2. Bioremediation is defined as a process of decontaminating soil and aquatic system by using plants, fungi or algae to absorb heavy metals.
3. Sambhar Salt Lake is India's largest inland salt water lake situated in Rajasthan, enriched with microorganisms.

OBJECTIVES OF RESEARCH WORK

1. Analysis of water and soil samples from different sites of Sambhar Lake in different seasons (summer, rainy and winter seasons) to study the following-

   (a) Physicochemical analysis including heavy metal profiling.
      (i) Color and pH
      (ii) Acidity and alkalinity
      (iii) Hardness
      (iv) Heavy metal analysis

(v) TSS (Total suspended Solids), TDS (Total Dissolved Solids) and TS (Total Solids)

(vi) Dissolved Oxygen (DO), Biological Oxygen Demand (BOD) and Chemical Oxygen Demand(COD)

(b) Morphological identification and population density study of algae in different seasons.

2. Analyzing suitability of collected Algae for the Bioremediation potential with heavy metals.
3. Bioremediation of the waste water samples containing heavy metals and screening of most efficient algal isolate with remediation potential.
4. Molecular identification of most efficient algae isolated.

SCOPE OF THE STUDY

At present scenario the accumulation of heavy metals by microalgae provide an advantage for bioremediation over other methods which are more costly and not environment friendly. Therefore, there is a need to improve the possibilities of accumulation of heavy metals in algae using suitable techniques to maximize bioaccumulation and the removal of toxic elements from the aquatic ecosystem.

LIMITATIONS

The limitations of work are –

1. The experimental work is confined to specific sites.
2. Although a number of halophilic bacteria are reported but this research is targeted by taking microalgae species in consideration.

SIGNIFICANCE OF THE STUDY

1. With the advantage of low cost raw material and no secondary pollution, algae is promising for purification of waste water containing heavy metals.
2. The objective significance of the proposed study is to isolate the most efficient naturally occurring algal species with high bioremediation capabilities of water bodies in Sambhar Lake and may be suggested for other salt lakes globally.

METHODOLOGY

Following procedure will be followed for the experimentation work:

- **Collection and analysis of water and soil samples including heavy metals.**

(i) **Collection of water and soil samples at different sites(during three seasons)**

This will be done at different sites of Sambhar salt lake and the water sample of industries effluents from 3-4 industries. The samples will be collected during three seasons; rainy season, winter season and summer season respectively. Water sample will be collected in 2 liters labelled bottles .These bottles will be placed at laboratory where sunlight is properly available .

(ii) **Physicochemical analysis including heavy metals**

The study of physicochemical parameters is very important to get idea about water quality. Water collected samples must be tested with different physico chemical parameters such as temperature, color, turbidity, pH, TDS, BOD,COD, dissolved oxygen, hardness, alkalinity and other characters by using standard methods of algal water sample(Trivedi, *et. al*, 2012).

Table 1: Different analytical water quality parameters with their analytical technique and Guideline value as per WHO and Indian standard (Patil *et, al*. 2012).

Sr. No.	Parameters	Technique used	WHO standard	Indian standard	EPA guidelines
01	Temperature	Thermometer	-	-	-
02	Color	Visual /color kit	-	5 Hazen units	-
03	Odour	Physiological sense	Acceptable	Acceptable	-
04	Electrical conductivity	Conductivity meter/ water analysis kit	-	-	2500 us/cm
05	pH	pH meter	6.5-9.5	6.5-9.5	6.5-9.5
06	Dissolved oxygen	Redox titration	-	-	-
07	Total hardness	Complexometric titration	200 ppm	300 ppm	<200 ppm
08	Alkalinity	Acid-Base titration	-	200 ppm	-
09	Acidity	Acid- Base titration	-	-	-
10	Ammonia	UV Visible spectrophotometer	0.3 ppm	0.5 ppm	0.5 ppm
11	Bi carbonate	Titration	-	-	-
12	Biochemical oxygen demand(B.O.D.)	Incubation followed by titration	6	30	5
13	Carbonate	Titration	-	-	-
14	(C.O.D.)	C.O.D. digester	10	-	40
15	Chloride	Argentometric titration	250 ppm	250 ppm	250ppm
16	Magnesium	Complexometric titration	150 ppm	30 ppm	
17	Nitrate	UV Visible spectrophotometer	45 ppm	45 ppm	50 mg/ml
18	Nitrite	UV Visible spectrophotometer	3 ppm	45 ppm	0.5 ppm
19	Potassium	Flame Photometer	-	-	-
20	Sodium	Flame Photometer	200 ppm	180 ppm	200 ppm
21	Sulphate	Nephelometer Turbidimeter	250 ppm	200 ppm	250 M

(iii) **Analysis of heavy metals**

The analysis of heavy metals is very important to evaluate the presence and concentration of heavy metals in water and soil samples. Different physicochemical tests can be used to analyze the heavy metals. After presumptive and confirmative tests for the heavy metal presence in the samples, the quantitative and qualitative analysis will be performed by Atomic Absorption Spectrophotometer.

(iv) **Morphological identification and population density study of algae in different seasons**

Algae are frequently found in polluted and unpolluted water and due to this behavior they are generally considered useful for bioremediation of water.

The water samples will be collected in the sterilized glass bottles. These sample bottles will be rinsed three times with source water to minimize the risk of external contamination before sampling. Algae will be identified and counted microscopically. 2 liters water samples will be collected from the selected locations. 15 ml samples will be taken and centrifuged at 3000 rpm at room temperature. The concentrate, ~20 µl of the sample will be taken onto glass slide, counted and enumerated under light microscope (40 X). Representative images will be taken at 100X magnification. (Prescott, 1956) Microalgae are unicellular and multicellular eukaryotic microorganisms that can be identified on the basis of their morphological and physiological characteristics like unicellular or filamentous, color, motility and reproductive structure.

- **Analyzing suitability of collected Algae for the Bioremediation potential with heavy metals.**

Different types of algae will be subjected for the bioremediation of water. These algae samples will be collected from water of different sites of Sambhar salt lake at different seasons. Microalgae will be isolated from Sambhar Salt Lake, Rajasthan, India. Single colony will be obtained by repeated sub-culturing on agar petri plates (Arun and Singh, 2014). All the cultures will be cultured in different medium. Cultures will be placed in culture room at 25 ± 2°C temperature, pH of 7.5 ± 1 under 24h day/night . Sub culturing will be followed after every 20 days Vidyalaxmi *et al,* (2019). Algae will be cultured in prescribed medium such as CFTRI Medium (Central Food Technological Research Institute) and BG-11 Medium (Blue Green) (Nyabuto *et al*, 2015).

- **Bioremediation of the waste water samples containing heavy metals and screening of most efficient algal isolate with remediation potential.**

300 ml of different water samples will be inoculated with 30 ml algal isolates of particular density in a flask and will be kept under illumination at 30ºC. Observation will be taken after an interval of 10 days and upto 30 days under aerobic conditions. For first 48 hrs. of incubation, the flask will be kept in a shaker at 100rpm for the purpose of uniform mixing of algae and effluents. Then periodically monitoring of the samples will be done for investing the physiochemical characteristics and biodegradability of the effluents. On the basis of Physico-chemical analysis the most efficient algal isolate will be screened on the basis of their reduction efficiency in BOD, DO and COD (Prabha *et al*, 2016).

- **Molecular identification of most efficient algae isolated.**

  The polymerase chain reaction (PCR) is a biomedical technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The study will be performed with the help of PCR amplification of specific nucleotides regions, like 16s or 18s rRNA genes. 16s rRNA present in Prokaryotes and 18s rRNA in Eukaryotes. This technique has been used for example, to determine the taxonomic composition of bio films- Blue green algae and some eukaryote unicellular algae (Droppo, *et. al*, 2007).

MONTH WISE PLAN OF WORK

1-30 months	Review of literature throughout the study
2-18 months	Physiochemical analysis of water and soil samples at every season and morphological identification and population density study of algae in different seasons
2-18 months	Analyzing suitability of collected algae for the Bioremediation potential
19-21 months	Bioremediation of waste water samples containing heavy metals and screening of most efficient algae
22-24 months	Molecular identification of most efficient algae
25-30 months	Thesis preparation

Total time (expected) = 30 months.

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